ELSEVIER

Journal of Chromatography B, 769 (2002) 55–64

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simultaneous determination of hydrocodone and hydromorphone in human plasma by liquid chromatography with tandem mass spectrometric detection

Yu-Luan Chen, Glenn D. Hanson, Xiangyu Jiang, Weng Naidong*

Department of Bioanalytical Chemistry, Covance Laboratories Inc., 3301 Kinsman Boulevard, Madison, WI 53704, USA

Received 2 October 2001; received in revised form 4 December 2001; accepted 13 December 2001

Abstract

A rapid, sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS–MS) method has been developed and validated for the simultaneous analysis of hydrocodone (HYC) and its metabolite hydromorphone (HYM) in human plasma. A robotic liquid handler and a 96-channel liquid handling workstation were used to aliquot samples, to add internal standard (I.S.), and to extract analytes of interest. A 96-well mixed-mode solid-phase cartridge plate was used to extract the analytes and I.S. The chromatographic separation was on a silica column (50×3 mm, 5-μm) with a mobile phase consisting of acetonitrile, water and trifluoroacetic acid (TFA) (92:8:0.01, v/v). The run time for each injection was 2.5 min with the retention times of approximately 2.1 and 2.2 min for HYC and HYM, respectively. The tandem mass spectrometric detection was by monitoring singly charged precursor→product ion transition 300→199 (m/z) for HYC, and 286→185 (m/z) for HYM. The validated calibration curve range was 0.100–100 ng/ml, based on a plasma volume of 0.3 ml. The correlation coefficients were greater than or equal to 0.9996 for both HYC and HYM. The low limit of quantitation (LLOQ) was 0.100 ng/ml for both HYC and HYM with signal-to-noise ratio (S/N) of 50 and 10, respectively. The deuterated analytes, used as internal standards, were monitored at mass transitions 303→199 (m/z) for HYC-d_3 and 289→185 (m/z) for HYM-d_3. The inter-day ($n=17$) precision of the quality control (QC) samples were ≤3.5% RSD (relative standard deviation) for HYC and ≤4.7% RSD for HYM, respectively. The inter-day accuracy of the QC samples were ≤2.1% RE (relative error) for HYC and ≤1.8% RE for HYM. The intra-day ($n=6$) precision and accuracy of the QC samples were ≤2.6% RSD and ≤3.0% RE for HYC, and ≤4.7% RSD and ≤2.4% RE for HYM. There was no significant deviation from the nominal values after a 5-fold dilution of high concentration QC samples by blank matrix. The QC samples were stable when kept at room temperature for 24-h or experienced three freeze–thaw cycles. The extraction recoveries were 86% for HYC and 78% for HYM. No detectable carryover was observed when a blank sample was injected immediately after a 2500 ng/ml sample that was 25-fold more concentrated than the upper limit of quantitation (ULOQ).

Keywords: 96-Well SPE; Hydrocodone; Hydromorphone

1. Introduction

Hydrocodone (4,5α-epoxy-3-methoxy-17-methyl-morphinan-6-one) is a semi-synthetic opioid analge-

*Corresponding author. Tel.: +1-608-242-2652; fax: +1-608-242-2735.

E-mail address: naidong.weng@covance.com (W. Naidong).

1570-0232/02/$ – see front matter
PII: S1570-0232(01)00616-X

sic related to but more potent than codeine and is frequently used for the relief of moderate pain [1]. Hydromorphone (4,5α-epoxy-3-hydroxy-17-methylmorphinan-6-one), a phenanthrene derivative, is another opioid analgesic with a greater potency than hydrocodone and is widely used in cancer patients [1]. The simultaneous analysis of hydrocodone (HYC) and hydromorphone (HYM) in biological matrices is of interest, because they might co-exist in the same formulation, and more importantly HYM is the active metabolite of HYC in the human body [1]. The chemical structures of HYC and HYM are shown in Fig. 1.

The measurement of HYM, together with morphine and/or metabolites, in biological fluids has been extensively studied [2–10]. Cone et al. [2] presented a gas chromatographic (GC) method to measure HYM and its metabolite in human urine. O'Connor et al. [3] reported an LC–electrochemical detection approach for the simultaneous determination of morphine, HYM, naltrexone and naloxone in plasma and urine. Saady et al. [4] developed a GC–MS assay for simultaneous quantification of morphine, codeine and HYM in blood or serum with a low limit of quantitation (LLOQ) of 80 ng/ml for HYM, which was too high for clinical studies. Wetzelsberger et al. [5] described an LC method that had a detection limit of 50 pg/ml but required 3 ml of sample. Lee et al. [6] presented a sensitive radioimmunoassay (RIA) method for the determination of HYM in plasma with an LLOQ of 50 pg/ml but required 1 ml sample. Bouquillon et al. [7] used solid-phase extraction and LC separation on a C_8 column with electrochemical detection for the simultaneous determination of morphine and HYM with an LLOQ of 1 ng/ml in plasma. Recently Naidong et al. [8] developed a sensitive normal-phase LC–MS–MS determination of HYM with an LLOQ of 50 pg/ml in human plasma based on a 1-ml sample. In the literature, only a few quantitative methods were reported for HYC [9–13]. Barnhart et al. [11] described a GC method to determine HYC in serum with a detection limit of 1 ng/ml. Hoffman et al. [12] presented a capillary GC method using a nitrogen-sensitive detector for HYC and carbinoxamine and achieved a detection limit of ca. 0.2 ng/ml using a 2-ml serum sample. Recently a liquid–liquid extraction combined with GC–MS was reported for the determination of morphine (1–1000 ng/ml), codeine (1–1000 ng/ml), HYC (1–1000 ng/ml) and 6-acetylmorphine (1–200 ng/ml) in blood [13]. A couple of RIA methods for the determination of HYC in body fluids were also published [9,10]. These published methods either required the preparation of a specific antibody or had a relatively higher LLOQ (a few nanogram per ml) and a narrow dynamic range. To our knowledge, there is no assay for the simultaneous determination of HYC and HYM in biological matrices.

In this presentation, a sensitive, rapid and specific method for the simultaneous quantification of HYC and HYM in human plasma is described. The method was validated for the analysis of both HYC and HYM in the 0.100–100 ng/ml range using a 0.3-ml plasma sample.

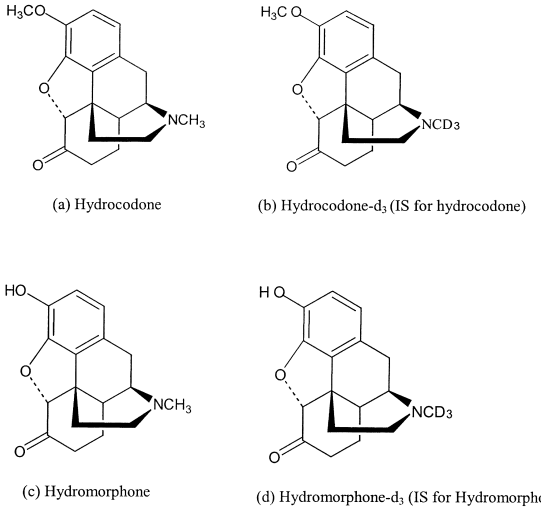

Fig. 1. Chemical structures of hydrocodone and hydromorphone and internal standards. (a) Hydrocodone, (b) hydrocodone-d_3, (c) hydromorphone, (d) hydromorphone-d_3.

2. Experimental

2.1. Chemicals and reagents

Hydrocodone bitartrate with a purity of 98.9% and hydromorphone hydrochloride with a purity of 100% were purchased from Sigma (St. Louis, MO, USA).

Hydrocodone-d_3, with an isotopic purity of 100%, was from Cambridge Isotope Labs (Andover, MA, USA) and hydromorphone-d_3 with an isotopic purity of 100% was from Cerilliant (Austin, TX, USA). The reference standards were used without further purification. The salt, moisture and impurity were corrected for when the stock solutions were prepared. Acetonitrile, methanol, and water were of LC grade and were from Fisher Scientific (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was from Sigma. Blank human plasma with K_3-EDTA as an anticoagulant was from Biochemed Pharmacologicals (Winchester, VA, USA).

2.2. Instrumentation

A Packard Multiprobe™ II robotic liquid handler (Meriden, CT, USA) was used for aliquoting plasma samples and adding internal standard solution. A VersaPlate™, with mixed-mode Certify™ cartridge, from Varian (Harbor City, CA, USA) was used for the solid-phase extraction (SPE). A Tomtec Quadra™ 96 workstation (Hamden, CT, USA) was programmed to process SPE sample cleanup. A 96-well evaporation system from Zymark (Hopkinton, MA, USA) was used for the evaporation of solvent. A Shimadzu HPLC system (Kyoto, Japan) including a solvent delivery system LC10ADVP, an autoinjector SIL10ADVP, a controller SCL10ADVP, and a column oven CTO10ASVP, combined with a PE Sciex API 3000 triple quadrupole mass spectrometer (Concord, ON, Canada) was used to separate and detect the analytes. A Betasil silica analytical column 50×3.0 mm with packing particle size of 5-μm and 100 Å pore size, from Keystone Scientific (Bellefonte, PA, USA) was employed. A Supelco 0.5-μm in-line prefilter was used to prevent any insoluble particles from entering the analytical column. Sciex *Analyst* software version 1.1 was used for the data acquisition and process. Peak areas of the chromatograms were integrated and the ratios of the analyte/I.S. were calculated for each analyte. A weighted $1/\text{concentration}^2$ linear regression was used to obtain calibration curves from standards. The regression equations of the calibration curves were then used to calculate the concentrations of quality control samples or practical clinical samples.

2.3. Chromatographic conditions

The mobile phase was comprised of acetonitrile, water, and TFA at a ratio of 92:8:0.01 (v/v). The flow-rate of the mobile phase was 0.700 ml/min. The column oven temperature was maintained at 25 °C and the injection volume was 10 μl. A mixture of acetonitrile, water and TFA with a ratio of 50:50:0.05 (v/v) was used as the injector wash solution. Autoinjector carryover was tested by injecting the upper limit of quantitation (ULOQ) standard, 100 ng/ml of each analyte in this case, followed by an extracted blank or reagent blank. A much higher concentration (2500 ng/ml), a 25-fold concentration of the ULOQ was also used to investigate the tolerance of the injector carryover. With the wash solution and the rinse mode set at 3, i.e. rinsed with wash solution both prior to and after each injection, there was no carryover observed for both analytes even after a 2500 ng/ml sample was injected onto the system.

2.4. Tandem mass spectrometric detection

A PE Sciex API 3000 triple quadrupole mass spectrometer with a turbo ionspray ionization source operated in a positive ion mode was used to detect the analytes. The monitoring pattern and conditions for each analyte were obtained and optimized by infusing ca. 0.2 μg/ml of the analyte in a solvent similar to the mobile phase. The multiple reaction mode (MRM) was used to obtain the total ion counts at different acquisition time points. A high voltage of 4.5 kV was applied to the sprayer. The turbo gas temperature was 450 °C and the auxiliary gas flow was 8 L/min. The flow settings of nebulizing gas, curtain gas, and collision gas at the instrument were 8, 10, and 6, respectively. All of the gas used in this experiment was nitrogen with a purity of >99.99% from AGA (Madison, WI, USA). The entrance potential (EP) was fixed at −10 V. All other parameters on mass spectrometer were optimized by the auto-tuning program of the *Analyst* software. The tuning ranges for these parameters included declustering potential (DP) from 0 to 100 V, focusing potential (FP) from 50 to 380 V, collision cell exit potential (CXP) from 0 to 60 V, and collision energy (CE) from 0 to 130 V. The optimized DP, FP, and

CXP were 46, 250 and 18 V for HYC; and 56, 300 and 12 V for HYM. The optimized (CE) for both HYC and HYM were 41 eV. Detection involved monitoring the fragmentation patterns m/z 300→199 for HYC and m/z 286→185 for HYM. The internal standards were monitored at m/z 303→199 for HYC-d_3 and m/z 289→185 for HYM-d_3. The dwell time was 200 ms for each analyte and 100 ms for each I.S. In this assay, both Q1 and Q3 quadrupoles were set at unit resolution. For each injection, the total acquisition time was 2.5 min.

2.5. Preparation of standards and quality control (QC) samples

Two separate weighings of each analyte were prepared and dissolved in methanol to make stock solutions. One stock solution was used to prepare calibration standards, the other one used to make QC samples. For the validation purposes, the stock solutions from the two weighings must have less than a 5% difference in the LC–MS–MS responses. The concentrations of both HYC and HYM stock solutions were 500 μg/ml. The calibration standards were prepared by adding appropriate amounts of the stock solutions into pooled blank plasma. Eight pooled standard concentrations were 0.100, 0.200, 0.500, 2.50, 10.0, 40.0, 80.0, and 100 ng/ml for both HYC and HYM. QC samples at concentrations of 0.300 (low), 10.0 (medium), and 75.0 (high) ng/ml of both analytes were prepared. Dilution QC samples at 200 ng/ml for HYC and HYM, and LLOQ QC samples at 0.100 ng/ml for both analytes, were also prepared. Standards and QC samples were aliquoted into 2-ml polypropylene vials (approximately 0.7 ml sample per vial) and stored at −20 °C.

2.6. Sample preparation

Human plasma samples were briefly vortex-mixed and centrifuged at 1200 *g* for 10 min. An aliquot of 0.300 ml of each sample was then transferred from the vial into a 1-ml 96-channel deep-well plate by the Packard Multiprobe™ II robotic liquid handler. Between pipettings, the Multiprobe tips were rinsed with water, 0.5% TFA in acetonitrile, and water. No carryover from sample transfer tips was observed. Twenty microliter of I.S. solution containing 100 ng/ml of both internal standards in 1:1 (v/v) methanol:water was added to each well except blanks by the Multiprobe. The sample plate was then moved to Tomtec Quadra™ 96 workstation to perform automated SPE using 25-mg Varian Certify™ mixed-mode cartridges. This cartridge plate was pre-conditioned by 0.8 ml of methanol followed by 0.8 ml of 5% acetic acid in water (v/v). Samples were diluted with 0.30 ml of 5% acetic acid and mixed well by aspirating–dispensing a volume of 0.30 ml for three cycles. The mixed samples were loaded onto the SPE cartridges. The cartridges were washed by 0.8 ml of 5% acetic acid followed by 0.8 ml of pure methanol. After drying cartridges at full vacuum for approximately 3 min, the analytes were eluted using two portions of 0.4 ml of 2% ammonium hydroxide in methanol (v/v) into another clean deep-well collection plate. The collected eluent was evaporated to complete dryness under a stream of nitrogen at 50 °C on a TurboVap™ 96 concentrator. The residues were reconstituted in 0.100 ml of acetonitrile–water–TFA (95:5:0.005, v/v).

2.7. Validation

The full validation experiment was designed following *''Guidance for Industry—Bioanalytical Method Validation''* recommended by the *Food and Drug Administration (FDA) of the United States* [14]. To evaluate the precision and accuracy of the assay, three validation batches were processed on three separate days. Each batch had one set of calibration standards and six replicates of QC samples at low, medium, and high concentration levels. One of the validation batches was also designed to examine the dilution integrity. In such batch six replicates of the dilution QC samples and six replicates of high QC samples (both using a partial volume of 60.0 μl), treated with a 5-fold dilution by blank plasma prior to extraction, were also run. The short-term stability of the plasma samples was also included in one validation batch. QC samples experiencing three cycles of freeze–thaw (freeze–thaw stability) or sitting at room temperature for approximately 24 h (bench-top stability) were processed together with standards and regular QC samples. One batch of the extracted samples was stored in the refrigerator (2–8 °C) for approximately 24 h then

re-injected onto the same system to determine the storage and injector stability of the processed samples. The sample injection sequence was randomly arranged through the entire curve except that the batch always started and ended with a calibration standard.

3. Results and discussion

3.1. LC–MS–MS

The electrospray ionization (ESI) mass spectra obtained by infusing HYC and HYM in 1:1 methanol–water are shown in Fig. 2a and c, respectively. The singly protonated molecular ions were observed for both HYC (m/z 300) and HYM (m/z 286). Under the optimized fragmentation conditions, several product ions were observed over the m/z range of 100–300. For HYC, the most abundant fragment was at m/z 199, as shown in Fig. 2b, and for HYM the most abundant peak was at m/z 185, as shown in Fig. 2d. To obtain the best sensitivity for the quantification, the transition patterns 300→199 and 286→185 were chosen to monitor HYC and HYM, respectively.

Naidong et al. [15] have discussed in detail the use of silica columns and aqueous–organic mobile phases for quantitative analysis of polar ionic analytes in biological fluid. The composition of the injection solution might produce substantial effects on the peak shape and sensitivity [16]. Therefore, a solution with weaker eluting strength than that of the mobile phase was highly recommended for use as a reconstitution solvent. In the case of normal-phase retention, weaker solution implied higher organic content in the solution. In this assay, a mixture of 95:5:0.005 acetonitrile–water–TFA (v/v) was used to dissolve the extracted residues. Fig. 3 show the mass chromatograms of the human plasma blank. Fig. 4 is the mass chromatograms of the extract of a low concentration QC plasma sample (0.300 ng/ml of both compounds) together with the internal standards. The retention times were 2.1 min for HYC and 2.2 min for HYM. A 0.1-min difference in retention time between two compounds is usually insufficient for separating them from each other using conventional LC detectors. However, tandem mass spectrometry used multi-channels for multi-

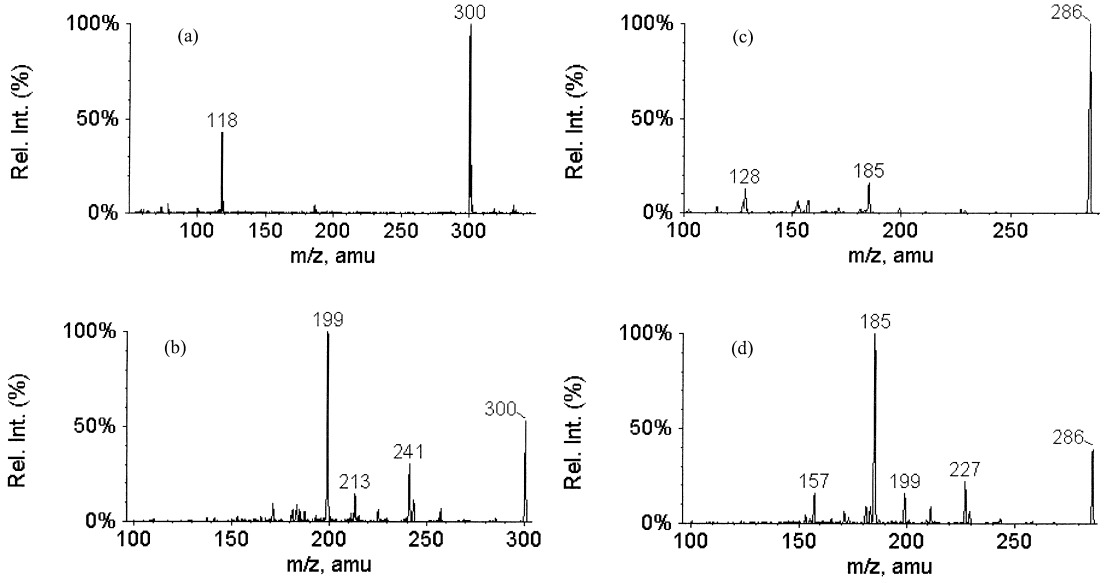

Fig. 2. ESI–mass spectra of hydrocodone and hydromorphone. (a) Hydrocodone, (b) MS–MS spectrum of hydrocodone, (c) hydromorphone, (d) MS–MS spectrum of hydromorphone.

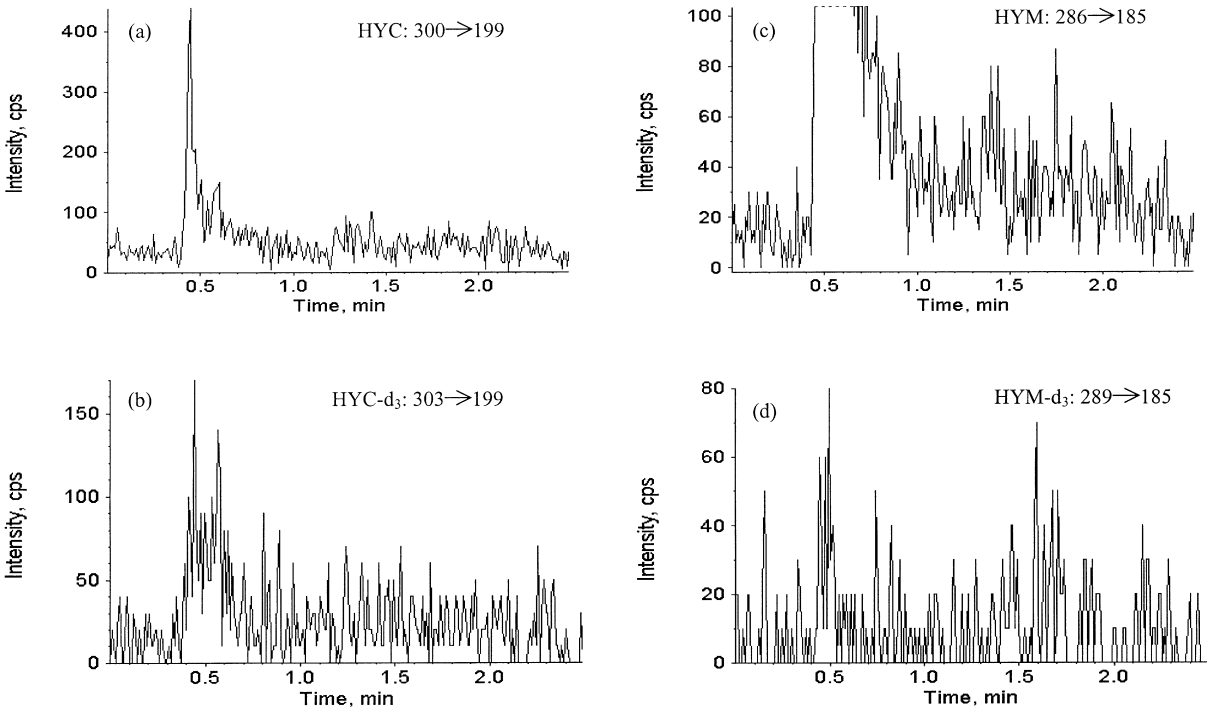

Fig. 3. Mass chromatograms of the extract of the matrix blank at different ion transitions. (a) 300→199 for HYC, (b) 303→199 for HYC-d_3, (c) 286→185 for HYM, and (d) 289→185 for HYM-d_3.

components (a channel for each specific analyte), and thus, gave excellent mass separation.

3.2. Specificity and selectivity

Six lots of blank plasma were tested for matrix effects and assay selectivity. For each lot of plasma, a plasma blank (free of analytes and I.S.), control 0 (plasma blank with I.S.), control 0.300 (fortified with 0.300 ng/ml of both analytes), and control 75.0 (fortified with 75.0 ng/ml both analytes) were used to investigate matrix interference and lot-to-lot matrix variation. The measured values and statistics are given in Table 1. For all six lots of plasma, the regions of the analytes and their I.S. peaks were found to be free of interference. When these six lots of plasma were separately fortified with the analytes at 0.300 ng/ml for HYC and HYM, the measured mean, RSD and RE were 0.304 ng/ml, 1.8% and +1.2% for HYC; and 0.298 ng/ml, 1.5% and −0.7% for HYM. For these plasma samples fortified with 75.0 ng/ml of HYC and HYM, the measured mean, RSD and RE were 74.3 ng/ml, 1.1% and −0.9% for HYC; and 74.1 ng/ml, 3.0%, and −1.2% for HYM, respectively. These results demonstrated that these six lots of plasma had no significant lot-to-lot matrix variation for both compounds.

A group of compounds such as fentanyl, naltrexone, naltrexol, 4-acetoidophenol, ibuprofen, propoxyphene, norpropoxyphene, morphine, oxymorphone, oxycodone, noroxycodone, hydromorphone-3-glucuronide (H3G), morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) were used to test the selectivity of the method. The tandem mass spectrometric detection was highly selective and specific. Only morphine was found to have interference to HYM. If morphine is co-dosed along with HYM, a better chromatographic separation for HYM from morphine will be needed. For the controlled clinical study samples, the measurement of HYM should not be influenced since morphine is not co-administered. No interference was observed for HYC.

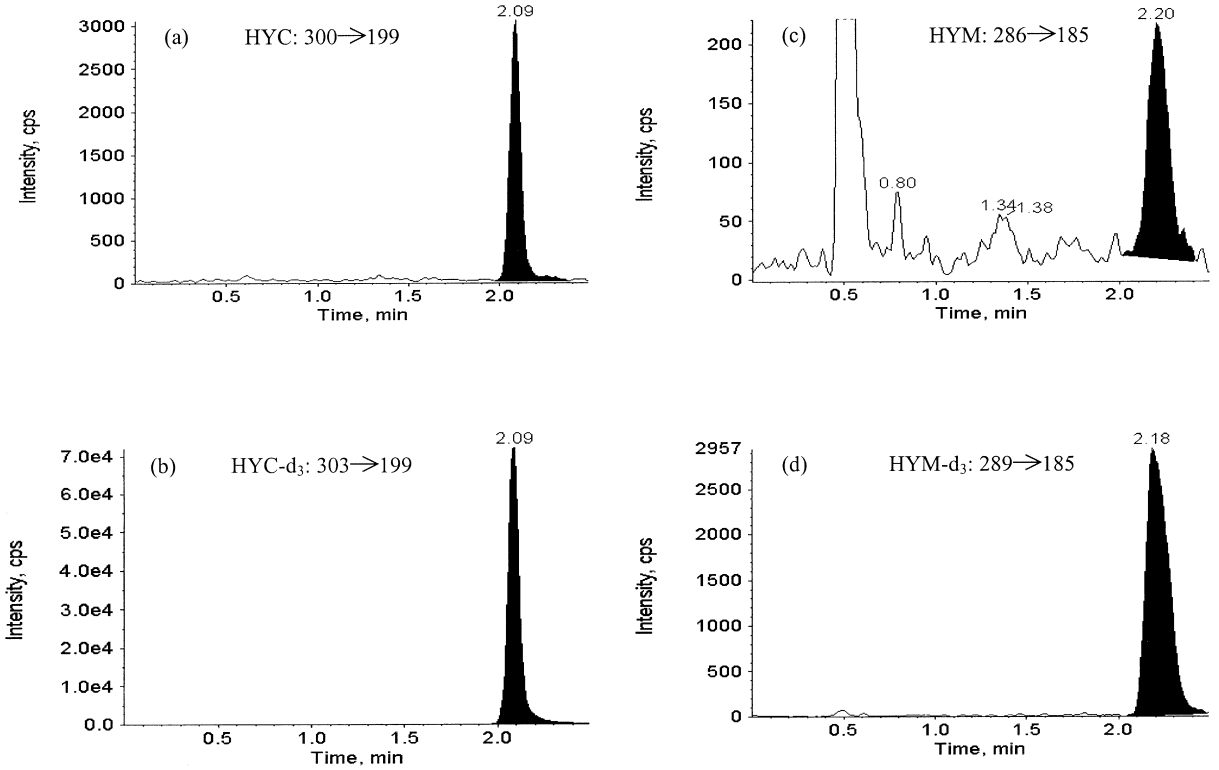

Fig. 4. Mass chromatograms of the extract of a quality control sample (0.3 ng/ml of both HYC and HYM) spiked with internal standards at different ion transitions. (a) 300→199 for HYC, (b) 303→199 for HYC-d_3, (c) 286→185 for HYM, and (d) 289→185 for HYM-d_3.

3.3. Sensitivity, linearity and recovery

The standard curve ranges were 0.100–100 ng/ml for both HYC and HYM when a 0.3-ml of plasma was used. Both compounds have a reliable LLOQ of 0.100 ng/ml in plasma. The signal-to-noise (S/N) ratio was ca. 50 for HYC and ca. 10 for HYM at the LLOQ concentration. HYC had a linear correlation coefficient better than or equal to 0.9998 and HYM had a linear correlation coefficient better than or

Table 1
Measured concentrations in individual plasma lots fortified with HYC and HYM

Lot	Hydrocodone (HYC)		Hydromorphone (HYM)	
	0.300 ng/ml	75.0 ng/ml	0.300 ng/ml	75.0 ng/ml
1	0.306	74.2	0.305	77.7
2	0.298	75.5	0.296	73.9
3	0.311	75.7	0.298	75.9
4	0.298	73.1	0.295	72.6
5	0.301	74.1	0.293	72.2
6	0.308	73.4	0.301	72.3
Mean (ng/ml)	0.304	74.3	0.298	74.1
RSD (%)	1.8	1.1	1.5	3.0
RE (%)	+1.2	−0.9	−0.7	−1.2

equal to 0.9996. The equations obtained from linear regression are as follows:

$$Y_{HYC} = 0.138X_{HYC} - 7.03 \times 10^{-5}$$

$$Y_{HYM} = 0.221X_{HYM} - 4.45 \times 10^{-4}$$

where X_{HYC} is the plasma concentration of HYC in ng/ml and Y_{HYC} is the ratio of the HYC/I.S. in the sample; similarly X_{HYM} is the plasma concentration of HYM in ng/ml and Y_{HYM} is the ratio of the HYM/I.S. in the sample. Recovery was determined by comparing peak areas of the analytes extracted from plasma with those of post-extraction plasma blanks fortified with the analytes. The results showed average recoveries of 86% for HYC and 78% for HYM.

3.4. Precision and accuracy

For all three validation curves, the results for all calibration standards showed a ≤2.5% RSD and ≤1.3% RE for HYC, and ≤2.6% RSD and ≤1.5% RE for HYM, respectively (data not shown). The precision and accuracy for the QC samples are given in Table 2. For HYC, the precision and accuracy at three concentration levels were typically ≤2.5% RSD and −3.0 to 0% RE for intra-day assays (n = 6), and 3.5% RSD and −1.5 to −2.1% RE for inter-day assays (n = 17 for low, medium and high QC samples). For HYM, the precision and accuracy at three concentration levels were ≤3.6% RSD and −1.0 to −2.4% RE for intra-day assays (n = 6), and 4.7% RSD and −0.4 to −1.8% RE for inter-day assays (n = 17 for low, medium and high QC samples). These results demonstrated that the present method has excellent precision and accuracy.

Table 3
Sample dilution integrity with a 5-fold dilution (n = 6)

QC sample (ng/ml)	Mean (ng/ml)	RSD (%)	RE (%)
Hydrocodone			
75.0	74.5	1.6	−0.7
200	198	3.3	−1.2
Hydromorphone			
75.0	71.5	5.2	−4.7
200	193	5.0	−3.3

3.5. Dilution integrity and stability

A 5-fold dilution for the dilution QC sample and high concentration QC sample by matrix blank prior to extraction was used to evaluate dilution integrity. Six replicates of partial volume dilution QC and high QC samples were processed in one of the validation batches. The data are described in Table 3. The results illustrated that taking partial volumes and diluting with matrix blank did not give significant deviation for the analytical data and the results met

Table 2
Precision and accuracy of quality control (QC) samples

QC sample (ng/ml)	Intra-day (n = 6)			Inter-day (n = 17)		
	Mean (ng/ml)	RSD (%)	RE (%)	Mean (ng/ml)	RSD (%)	RE (%)
Hydrocodone						
0.100	0.100	2.6	0.0	–	–	–
0.300	0.296	2.5	−1.3	0.294	2.9	−2.1
10.0	10.0	2.1	0.0	9.82	2.6	−1.8
75.0	72.8	1.9	−3.0	73.9	3.5	−1.5
Hydromorphone						
0.100	0.100	4.7	+0.4	–	–	–
0.300	0.297	3.6	−1.0	0.297	4.5	−1.0
10.0	9.82	2.9	−1.8	9.82	3.6	−1.8
75.0	73.2	3.2	−2.4	74.7	4.7	−0.4

the acceptance criteria (≤15% RSD and ≤15% RE) suggested by the FDA guidance for industry [14].

The short-term stability experiments were designed to test effects of freeze–thaw cycles, short-term storage at room temperature, and during analysis when extracted samples may be refrigerated. These experiments were performed as described in Section 2.7. All stability results are summarized in Table 4. Three freeze–thaw cycles and a 24-h room temperature storage for QC samples had no substantial effect on the analysis. Storing the extracted samples at 2–8 °C prior to injection did not affect the quantitative determination of HYC and HYM in samples.

3.6. Automation

The capability of rapid analysis by LC–MS–MS requires fast sample preparation to achieve a real high-throughput sample analysis. Automation is critical to meet the above needs. Several research groups have carried out studies in such fields [17–22]. The automation in this study included aliquoting sample, adding I.S. solution, and performing solid-phase extraction. The first two steps were performed using a Packard Multiprobe II robotic liquid handler with four tips, and the SPE cleanup was carried out using a Tomtec Quadra 96 workstation. The Multiprobe has excellent precision and accuracy and is suitable for aliquoting samples from vials to 96-well plates. The Tomtec can handle as many as 96 samples at a time. The precision and accuracy of the validation data confirmed the excellent performance of the combined above two automation systems.

4. Conclusion

A rapid, sensitive, and automated LC–MS–MS method for the simultaneous determination of HYC and HYM in human plasma was developed and validated. This method used mixed-mode SPE cartridge plate for sample preparation and a silica column coupled with MS–MS for separation and detection. The standard curve ranges for both analytes were 0.100–100 ng/ml in human plasma. The low limit of quantitation was 0.100 ng/ml for both HYC and HYM using only 0.3 ml plasma sample. The inter-day ($n=17$) precision of the quality samples were ≤3.5% RSD for HYC and ≤4.7% RSD for HYM. The inter-day accuracy of the QC samples were ≤2.1% RE for HYC, and ≤1.8% RE for HYM. Use of automation in 96-well format provided a high-throughput sample preparation. The described method has been applied to the quantification of hydrocodone and hydromorphone in human plasma samples from clinical trials.

References

[1] K. Parfitt, 32nd ed, Martindale: The Complete Drug Reference, The Pharmaceutical Press, London, 1999, p. 43 and 67.
[2] E.J. Cone, B.A. Phelps, C.W. Gorodetzky, J. Pharm. Sci. 66 (1977) 1709.
[3] E.F. O'Connor, S.W. Cheng, W.G. North, J. Chromatogr. 491 (1989) 240.
[4] J.J. Saady, N. Narasimhachari, R.V. Blanke, J. Anal. Toxicol. 6 (1982) 235.
[5] N. Wetzelsberger, P.W. Lucker, W. Erking, Arzneimittel-Forschung 36 (1986) 1707.

Table 4
Stability data (Unit: % of theoretical value)

QC sample (ng/ml)	Three freeze–thaw cycles ($n=6$)	Room temperature storage 24 h ($n=6$)	Processed sample refrigerated 24 h ($n=6$)
Hydrocodone			
0.300	95.0–101	96.7–105	93.7–100
10.0	96.4–102	99.2–104	94.7–98.5
75.0	96.9–99.3	98.3–101	91.2–99.2
Hydromorphone			
0.300	95.0–103	92.0–103	97.3–111
10.0	91.3–101	91.7–98.7	93.5–101
75.0	92.4–97.9	92.5–99.3	92.0–102

[6] J.W. Lee, J.E. Pedersen, T.L. Moravetz, A.M. Dzerk, K.V. Shepard, J. Pharm. Sci. 80 (1991) 284.
[7] A.I. Bouquillon, D. Freeman, D.E. Moulin, J. Chromatogr. 577 (1992) 354.
[8] W. Naidong, X. Jiang, K. Newland, R. Coe, P. Lin, J. Lee, J. Pharm. Biomed. Anal. 23 (2000) 697.
[9] J.W.A. Findlay, E.C. Jones, R.M. Welch, Drug Metab. Dispos. 7 (1979) 310.
[10] I.L. Honigberg, J.T. Stewart, J. Pharm. Sci. 69 (1980) 1171.
[11] J.W. Barnhart, W.J. Caldwell, J. Chromatogr. 130 (1977) 234.
[12] D.J. Hoffman, M.J. Leveque, T. Thomson, J. Pharm. Sci. 72 (1983) 1342.
[13] A.J. Jenkins, E.S. Lavins, J. Anal. Toxicol. 22 (1998) 173.
[14] Food and Drug Administration of the United States, Guidance for Industry—Bioanalytical Method Validation (May, 2001), US Department of Health and Human Services, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), http://www.fda.gov/cder/guidance/index.htm
[15] W. Naidong, W.Z. Shou, Y.-L. Chen, X. Jiang, J. Chromatogr. B 754 (2001) 387.
[16] W. Naidong, Y.-L. Chen, W.Z. Shou, X. Jiang, J. Pharm. Biomed. Anal. 26 (2001) 753.
[17] J.P. Allanson, R.A. Biddlecombe, A.E. Jones, S. Pleasance, Rapid Commun. Mass Spectrom. 10 (1996) 811.
[18] J. Janiszewski, R.P. Schneider, K. Hoffmaster, M. Swyden, D. Wells, H. Fouda, Rapid Commun. Mass Spectrom. 11 (1997) 1033.
[19] H. Simpson, A. Berthemy, D. Buhrman, R. Burton, J. Newton, M. Kealy, D. Wells, D. Wu, Rapid Commun. Mass Spectrom. 12 (1998) 75.
[20] N.H. Huang, J.R. Kagel, D.T. Rossi, J. Pharm. Biomed. Anal. 19 (1999) 613.
[21] W.Z. Shou, X. Jiang, B.D. Beato, W. Naidong, Rapid Commun. Mass Spectrom. 15 (2001) 466.
[22] W.Z. Shou, M. Pelzer, T. Addison, X. Jiang, W. Naidong, J. Pharm. Biomed. Anal 27 (2002) 143.